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TITLE: Identification of Apoptosis Genes Induced by the
Human Prolactin Antagonist, hPRL-G129R, in Several
Breast Cancer Cell Lines

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Introduction

To gain insight into the molecular basis of human prolactin (hPRL) antagonist induced apoptosis, we compared the differential gene expression profile of four human breast cancer cell lines following treatment with hPRL and its antagonist (hPRL-G129R). Among the genes identified, the *bcl-2* gene was of particular interest. We found that *bcl-2* mRNA was up regulated in three of the four cell lines that were treated with hPRL. To further confirm these results, real time RT-PCR and ELISA analyses were used to detect *bcl-2* mRNA and Bcl-2 protein, respectively, in eleven different breast cancer cell lines after hPRL or hPRL-G129R treatment. Our data suggest that Bcl-2 is up regulated in response to hPRL stimulation and is competitively inhibited by hPRL-G129R in the majority of the cell lines tested. We propose that the anti-apoptotic role of hPRL in breast cancer is mediated, at least in part, through regulation of Bcl-2. We advanced these studies by using real time RT PCR to measure *bax* gene expression in these same eleven human breast cancer cell lines. We also measured *bax* and *bcl-2* gene expression in six groups of transgenic mice of three different ages, one expressing PRL and the other expressing G129R, as well as litter-matched nontransgenics. We found that *bax* mRNA was significantly up regulated in

Introduction (continued)

five of the eleven G129R treated cell lines, as well as in the nine month age group of G129R transgenic mice. To further confirm these results, Bax and Bcl-2 proteins were analyzed by Western blot methods in selected cell lines and from mammary gland tissue homogenates of transgenic mice. Bax/Bcl-2 ratios were highest in the six month group of G129R transgenics, and lowest in the six month group of PRL transgenics. We expanded our findings by examining the release of a downstream Bax-induced protein, cytochrome c, a hallmark protein of apoptosis, in selected cell lines and in tissue homogenates of all transgenic mice. Again, cytochrome c levels were highest in the six month G129R transgenic group. Our data suggest that Bax is up regulated in response to G129R stimulation both *in vitro* and *in vivo* and that its expression is closely linked to cytochrome c release. We propose that apoptosis in these model breast cancer systems is in part driven by the up regulation of Bax at both the mRNA and protein levels, leading to the ultimate release of cytochrome c.

Body: Research accomplishments associated with each task outlined in the approved Statement of Work.

Specific Aim I: To identify genes specific to human prolactin antagonist-induced apoptosis in human breast cancer cell lines.

In this study, we examined the profile of apoptosis related genes expressed by four human breast cancer cell lines upon treatment with either hPRL or hPRL-G129R, using a newly described technique called suppression subtractive hybridization (SSH) in combination with cDNA microarrays. It was found that *bcl-2* gene expression was increased following treatment of breast cancer cells with hPRL in both estrogen receptor (ER) positive cell lines and one of two ER negative cell lines tested.

We compared all relevant apoptosis related genes expressed in human breast cancer cells. Treatment of T-47D and MCF-7 cells with hPRL up-regulated *bcl-2* expression. In contrast, T-47D cells treated with hPRL-G129R exhibited a strong up-regulation of caspases, such as caspase 3, 4, 7, 9 and 10. The Bcl-2 binding protein, BNIP₃, was expressed in all but the MDA-MB-468 after hPRL-G129R treatment. In the T-47D cells, the genes related to death receptors such as Serine-Threonine Kinase 1, DAXX, Tumor Necrosis Factor-Related Apoptosis Inducing Ligand and Death Domain Receptor 3 were up-regulated after hPRL-G129R treatment, although the gene for caspase 8, which is normally associated with death receptors, was not. In MCF-7 cells the gene *BAD*, an important member of the Bcl-2 family of proteins, was differentially expressed after hPRL-G129R treatment. There was no evidence of caspase expression in MCF-7 cells after hPRL-G129R treatment.

Specific Aim II: Use of a quantitative method of RT-PCR (reverse transcription “real time” PCR) to determine the mRNA copy number of the apoptotic-related gene we identified as most critical, *bcl-2*, in eleven breast cancer cell lines.

To confirm the evidence linking hPRL and Bcl-2, a quantitative method of RT-PCR was used to measure *bcl-2* mRNA expression levels, in eleven human breast cancer cell lines after treatment with hPRL or hPRL-G129R. The data from these studies suggest that hPRL acts as an apoptosis inhibitor by increasing the expression of Bcl-2 in

human breast cancer, and that hPRL-G129R competitively inhibits Bcl-2 induction by hPRL (Figures 1 and 2; Table 1).

Additional Aim III: Use of a quantitative method of RT-PCR (reverse transcription “real time” PCR) to determine the mRNA copy number of the apoptotic-related gene *bax*, in eleven breast cancer cell lines, as well both *bax* and *bcl-2* levels in mammary gland tissue of 3 age groups of PRL and G129R transgenic mice.

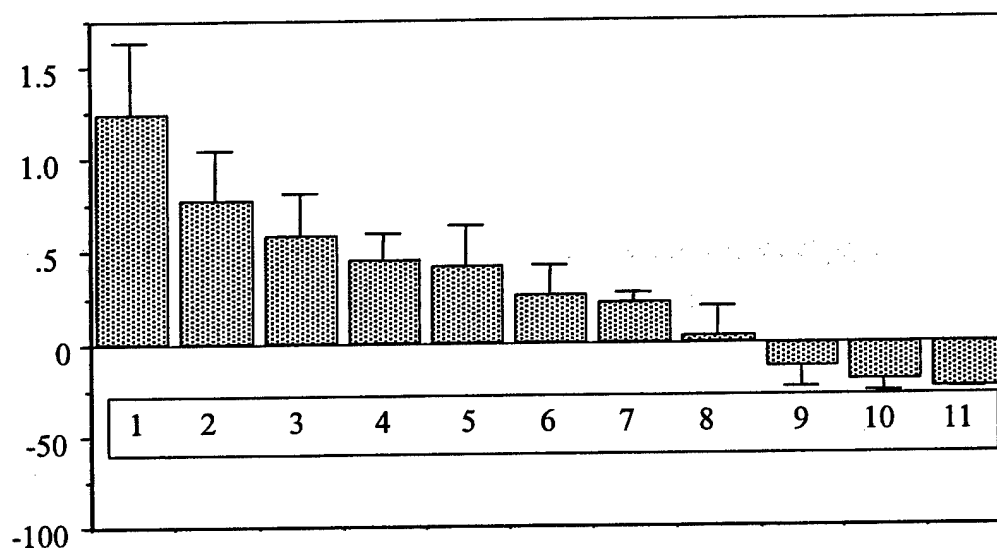
We found that *bax* mRNA levels were significantly up regulated in five of the eleven G129R treated cell lines, as well as in the nine month age group of G129R transgenic mice (Figure 3).

Additional Aim IV: Western blots analyses of levels of Bax, Bcl-2 and cytochrome c in human breast cancer cell lines and in mammary gland tissue of PRL and G129R transgenic mice.

We found that at the protein level, Bax/Bcl-2 ratios were highest in the six month group of G129R transgenics, and lowest in the six month group of PRL transgenics. Moreover, cytochrome c levels were highest in the six month G129R transgenic group, suggesting that apoptosis in these model breast cancer systems is, in part, driven by the up regulation of Bax at both the mRNA and protein levels, leading to the ultimate release of cytochrome c (Figure 4).

Figure 1: Real time quantitative measurement of *bcl-2* mRNA levels in eleven breast cancer cell lines in response to 48-h treatments with hPRL (500 ng/ml). Levels are represented as the relative change from the untreated controls (=0), and numbers are presented as mean \pm SE. All samples were normalized to equivalent levels of β -actin mRNA.

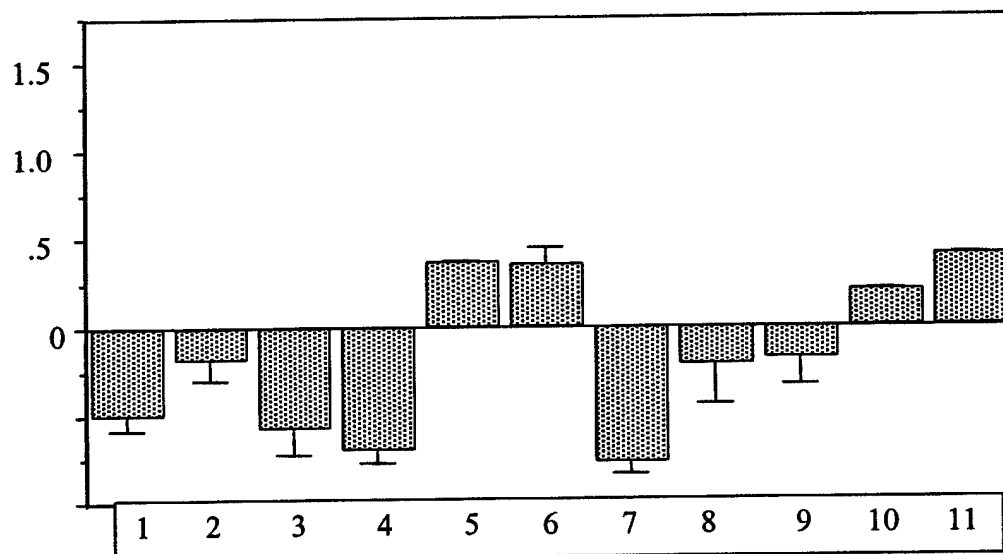
FIGURE 1



CELL LINES: 1. MCF-7; 2. MDA-MB-134; 3. T-47D; 4. BT549; 5. MDA-MB-436; 6. MDA-MB-468; 7. MDA-MB-157; 8. MDA-MB-453; 9. BT474; 10. MDA-MB-231; 11. BT483.

Figure 2: Real time quantitative measurement of *bcl-2* mRNA levels in eleven breast cancer cell lines in response to 48-h treatments with hPRL-G129R (500 ng/ml). Levels are represented as the relative change from the untreated controls (=0), and numbers are presented as mean \pm SE. All samples were normalized to equivalent levels of β -actin mRNA.

FIGURE 2



CELL LINES: 1. MCF-7; 2. MDA-MB-134; 3. T-47D; 4. BT549; 5. MDA-MB-436; 6. MDA-MB-468; 7. MDA-MB-157; 8. MDA-MB-453; 9. BT474; 10. MDA-MB-231; 11. BT483.

Table 1 summarizes the *bcl-2* levels from multiple quantitative real time RT-PCR runs relative to normalized levels of β -actin. The data are presented as levels of *bcl-2* in all eleven cell lines treated with hPRL, hPRL-G129R or a combination of hPRL and hPRL-G129R, and were compared to levels of *bcl-2* in the untreated controls.

In MCF-7 and MDA-MB-134 cells, hPRL treatment resulted in a highly significant ($P < 0.01$) up-regulation of *bcl-2* message, while in BT-549 and T-47D cells the percent change was significant ($P < 0.05$). In the remaining seven cell lines, *bcl-2* message levels were not significantly different from the untreated controls. Treatment with the antagonist resulted in significantly ($P < 0.05$) decreased expression of *bcl-2*

message in four of the cell lines (MCF-7, T-47D, BT-549 and MDA-MB-157) and no significant change in the other cell lines. A modest increase in *bcl-2* message expression was observed in four cell lines (MDA-MB-436, MDA-MB-468, MDA-MB-231, BT-483) following hPRL-G129R treatment. In seven cell lines a combination treatment of hPRL-G129R and hPRL resulted in lower levels of *bcl-2* expression than the hPRL treatment alone (MCF-7, T-47D, MDA-MB-134, MDA-MB-453, BT-474, MDA-MB-231 and BT-483). The combination treatment significantly ($P<0.05$) reduced *bcl-2* expression levels in MCF-7 and T-47D cell lines, whereas MDA-MB-134, MDA-MB-453, BT-474, MDA-MB-231 and BT-483 cells show an insignificant decrease in the level of Bcl-2.

Table 1 Fold difference of *bcl-2* message of treatments over untreated cells.

Cell line	PRL ^a	G129R ^b	PRL+G129R ^c
MCF-7	2.25 ± 0.19	0.50 ± 0.08	0.45 ± 0.15
MDA-MB-134	1.78 ± 0.27	0.82 ± 0.12	0.76 ± 0.17
T47-D	1.59 ± 0.29	0.43 ± 0.15	0.24 ± 0.03
BT549	1.46 ± 0.14	0.30 ± 0.07	1.41 ± 0.38
MDA-MB-436	1.42 ± 0.28	1.38	2.77
MDA-MB-468	1.27 ± 0.15	1.26 ± 0.09	1.44 ± 0.09
MDA-MB-157	1.22 ± 0.05	0.33 ± 0.07	1.26
MDA-MB-453	1.04 ± 0.16	0.78 ± 0.22	0.75
BT474	0.87 ± 0.11	0.82 ± 0.15	0.88 ± 0.27
MDA-MB-231	0.79 ± 0.06	1.21	0.43
BT483	0.75	1.41	0.55

TABLE 1: Numbers represent real time RT-PCR data correlating to Figures 1 and 2 and represent *bcl-2* message levels. Cells were either treated with hPRL (500 ng/ml) or hPRL-G129R (500 ng/ml). Combination treatment (PRL+G129R) is as follows: 200 ng/ml PRL + 1000 ng/ml G129R for 48-h. All values are represented as fold change over the untreated controls and are mean ± SE. a, n=2-5; b, n=2-4; c, n=2-3.

Using the mean values for the Bax and Bcl-2 mRNA levels, the bax/bcl-2 ratios were calculated for the G129R treatments and the bcl-2/bax ratios for PRL treatments were calculated in these eleven cell lines. The bax/bcl-2 ratios for G129R treatment indicated a

major increase in bax levels over bcl-2 levels in seven of the eleven lines, with the two cell lines having the highest PRL receptor levels (T47D and BT134) showing the largest ratios (Figure 3).

Mammary gland tissue in three age groups (3M, 6M and 9M) of transgenic mice expressing either PRL or G129R was analyzed for levels of Bax, Bcl-2 and cytochrome c proteins. The non-transgenic (control group) littermates were analyzed simultaneously. All samples were normalized to levels of α -tubulin, as quantified by densitometric scanning. At three months, the levels of Bax and Bcl-2 are very similar between the two groups of transgenics, although the G129R transgenics are already expressing slightly more Bax, and PRL transgenics slightly more Bcl-2, at the protein level. However, by six months, the G129R transgenics are expressing considerably more Bax protein, than the corresponding PRL transgenics (Figure 4). Moreover, by six months, the level of cytochrome c expression has markedly increased in the G129R transgenic animals.

Figure 3: Bax/Bcl-2 mRNA ratios from real time RT-PCR data calculated from the mean value of each cell line as fold change from the untreated controls; 48h treatment of eleven human breast cancer cell lines with 500 ng/ml of G129R.
1 = no change in ratio.

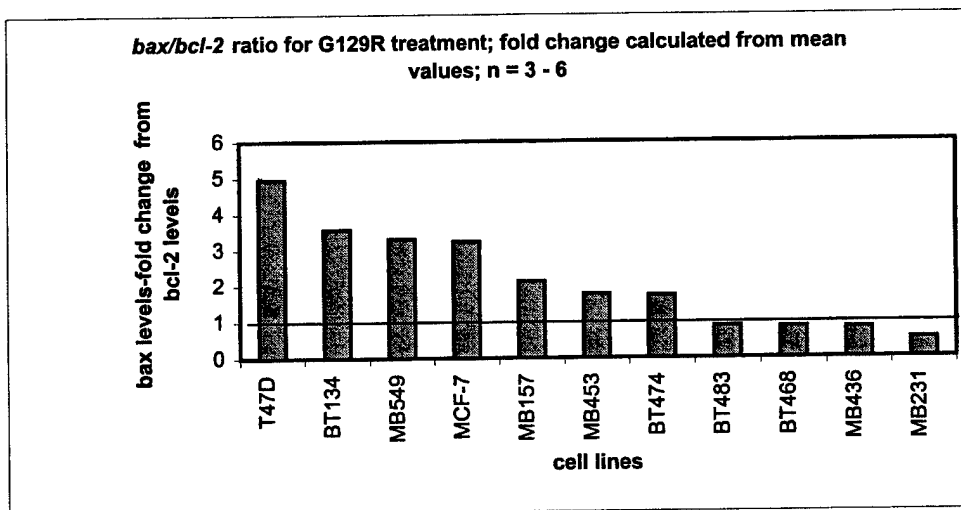
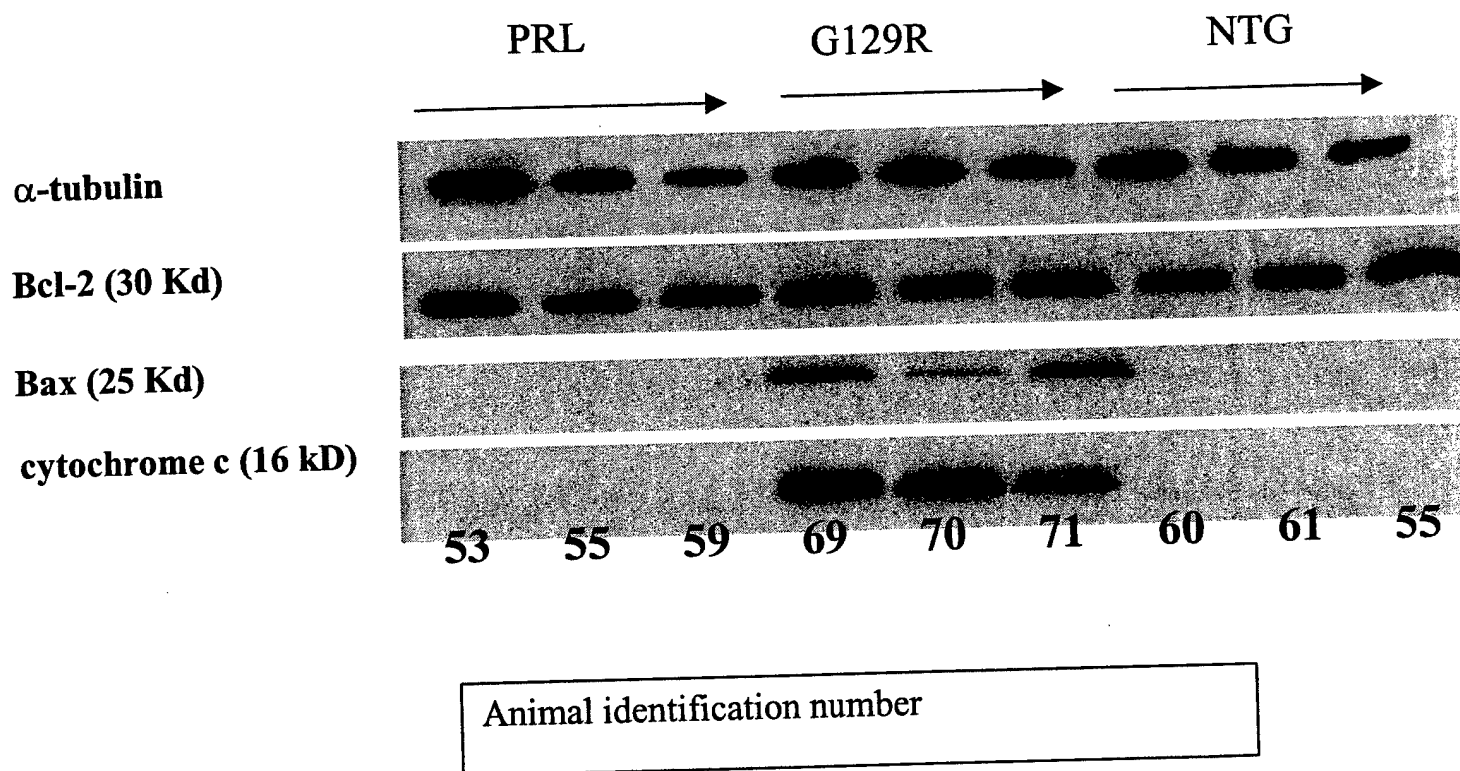


Figure 4: Western blot analyses from 6 month PRL and G129R transgenics and age-matched non-transgenic littermates. Fifty micrograms of protein were loaded per well. Blots were hybridized first to an anti-Bcl-2 antibody and stripped. In successive hybridizations and strippings, blots were then analyzed for Bax, cytochrome c, and α -tubulin. Three littermates were chosen for each transgenic grouping. One or two non-transgenic (NTG) littermates from each litter were chosen for the non-transgenic grouping.



In summary, a number of apoptosis-related genes that are differentially expressed following treatment with either hPRL or hPRL-G129R have been identified for four different breast cancer cell lines. These data will allow for future studies of specific genes that are involved with cellular proliferation or apoptosis in human breast cancer. By focusing on *bax* and *bcl-2* mRNA expression in response to hPRL and hPRL-G129R treatment in eleven cell lines, we provide further evidence that the anti-apoptotic effects of hPRL in breast cancer are likely mediated through the up regulation of Bcl-2 and down-regulation of Bax. Thus, apoptosis in these breast cancer model systems is driven, at least in part, by the simultaneous down-regulation of Bcl-2 and the up-regulation of Bax at both the mRNA and protein levels, regulation that leads to the ultimate release of cytochrome c. In total, these data support the growing body of evidence of the potential importance of this prolactin antagonist for use in the treatment of breast cancer.

Key Research Accomplishments

- Comparison of differential gene expression of four human breast cancer cell lines in response to treatment with human prolactin (hPRL) and its antagonist (hPRL-G129R) using a cDNA microarray representing 204 apoptosis-related genes
- Determination of the importance of the *bax* and *bcl-2* genes in the response to treatment with (hPRL) or its antagonist (hPRL-G129R) by human breast cancer cell lines
- Quantitative analysis of levels of *bax* and *bcl-2* mRNA in eleven human breast cancer cells by real time RT-PCR in response to treatment with (hPRL), its antagonist (hPRL-G129R), or a combination of treatments
- Statistical analyses of levels of *bax* and *bcl-2* mRNA of eleven human breast cancer cells in response to treatment with (hPRL), its antagonist (hPRL-G129R), or a combination of treatments
- Quantitative analysis by real time RT-PCR of *bax* and *bcl-2* gene expression in six groups of transgenic mice of three different ages, one expressing PRL and the other expressing G129R, as well as age-matched nontransgenics; statistical analyses as described above
- Western blot determination of Bax, Bcl-2 and cytochrome c levels in eleven human breast cancer cell lines and six groups of transgenic mice of three different ages, one expressing PRL and the other expressing G129R, as well as age-matched nontransgenics; determination of Bax/Bcl-2 ratios in these transgenics from densitometric scans; statistical analyses as described above

Key Research Accomplishments (continued)

- Western blot determination of the release of a downstream Bax-induced protein, cytochrome c, a hallmark protein of apoptosis, in selected cell lines and in tissue homogenates of all transgenic mice

Reportable Outcomes

Manuscripts:

1. Beck MT, Peirce,SK, and Chen WY. (2002). **Regulation of *bcl-2* gene expression in human breast cancer cells by prolactin and its antagonist, hPRL-G129R.** *Oncogene* 21, 5047-5055.
2. Chen NY, Holle L, Li W, Peirce SK, Beck MT and Chen WY (2002). ***In vivo* studies of the anti-tumor effects of a human prolactin antagonist, hPRL-G129R.** *Int. J. Oncology* 20: 813-818.
3. Peirce SK and Chen WY (2001).**Quantification of prolactin receptor mRNA in multiple human tissues and cancer cell lines by real time RT-PCR.** *J. Endocrinology*, 171, R1-R4
4. Peirce SK and Chen WY (2003): **A human prolactin antagonist, G129R, upregulates Bax gene and protein expression leading to cytochrome c release in human breast cancer model systems.** Manuscript in progress; to be submitted to *Oncogene*.

Abstracts and Poster Sessions

Using A Combination Approach of Human Prolactin Antagonist and Herceptin for the Treatment of Her2-Overexpressing Breast Cancer.

Michele L. Scotti, Susan K. Peirce, Karl J. Franek and Wen Y. Chen.
(American Association of Cancer Research Annual Meeting, Washington DC, 10-14 July 2003).

Regulation of Bcl-2 Expression by hPRL and Its Antagonist, hPRL-G129R, in Human Breast Cancer Cell Lines.

Susan K. Peirce. Michael T. Beck and Wen Y. Chen

Endo 2002 (The Endocrine Society's 84th Annual Meeting); San Francisco, June 19-22, 2002

Chosen for inclusion in the ENDO 2002 Research Summaries Book (RSB). Each year, the Media Advisory Committee (MED) and the Public Affairs staff of The Endocrine Society identify approximately 125 newsworthy abstracts to include in the RSB.

From An Antagonist Back To An Agonist: Two Wrongs Do Make A Right.

J.F. Langenheim, M.T.Beck, S.K.Peirce, L.Holle and W.Y.Chen
Endo 2002 (The Endocrine Society's 84th Annual Meeting); San Francisco, June 19-22, 2002

Chosen for both oral and poster presentation.

Real Time RT PCR Analysis of Relative Prolactin Receptor (PRLR) Levels in Human Cancer Cell Lines.

S.K. Peirce, R.B. Westberry and Wen Y. Chen.

Endo 2001(The Endocrine Society's 83rd Annual Meeting), Denver, June 20-23, 2001

Degree

Ph.D, May 2003

Clemson University; Department of Genetics and Biochemistry

Advisor: Dr. Wen Y. Chen

Employment and Research Opportunities

Postdoctoral Fellowship offer, in the laboratory of Dr. Ruth Lupu, Director, Breast Cancer Translational Research.

Northwestern University Healthcare Institute and Northwestern University Medical School.

Evanston, Illinois, 60201.

Conclusions

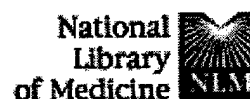
Our data will allow for future studies of specific genes that are involved with cellular proliferation or apoptosis in human breast cancer. By focusing on *bcl-2* mRNA expression in response to hPRL and hPRL-G129R treatment in eleven cell lines, we provide further evidence that the anti-apoptotic effects of hPRL in breast cancer are likely mediated through the up-regulation of Bcl-2. Moreover, we have shown in this study that Bax is up-regulated in response to G129R stimulation both *in vitro* and *in vivo*, and that this expression of Bax is closely linked to cytochrome c release. It is generally accepted that for cancer therapy one should not design an approach based solely upon increasing death signals, such as chemotherapeutics. Rather, a two-fold approach combining chemotherapeutics with removal of survival factors will result in a more efficient treatment. hPRL-G129R employs such a two-pronged attack in these model breast cancer systems. Our data regarding hPRL-G129R further strengthens its potential therapeutic role in breast cancer therapy, especially in combination with other chemotherapeutics.

References

- Peirce SK and Chen WY (2003). **A human prolactin antagonist, G129R, upregulates Bax gene and protein expression leading to cytochrome c release in human breast cancer model systems.** Manuscript in progress; to be submitted to *Oncogene*.
- Beck MT, Peirce,SK, and Chen WY. (2002). **Regulation of *bcl-2* gene expression in human breast cancer cells by prolactin and its antagonist, hPRL-G129R.** *Oncogene* 1; 2 (33):5047-5055.
- Chen NY, Holle L, Li W, Peirce SK, Beck MT and Chen WY (2002). ***In vivo* studies of the anti-tumor effects of a human prolactin antagonist, hPRL-G129R.** *Int. J. Oncology* 20: 813-818.
- Peirce SK and Chen WY (2001).**Quantification of prolactin receptor mRNA in multiple human tissues and cancer cell lines by real time RT-PCR.** *J. Endocrinology*, 171, R1-R4

Appendices

See attached abstracts.



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1: Beck MT, Peirce SK, Chen WY.

Related Articles, Li

Regulation of bcl-2 gene expression in human breast cancer cells by prolactin and its antagonist, hPRL-G129R.

Oncogene. 2002 Aug 1;21(33):5047-55.

PMID: 12140755 [PubMed - indexed for MEDLINE]

2: Chen NY, Holle L, Li W, Peirce SK, Beck MT, Chen WY.

Related Articles, Li

In vivo studies of the anti-tumor effects of a human prolactin antagonist, hPRL-G129R.

Int J Oncol. 2002 Apr;20(4):813-8.

PMID: 11894130 [PubMed - indexed for MEDLINE]

3: Peirce SK, Chen WY, Chen WY.

Related Articles, Li

Quantification of prolactin receptor mRNA in multiple human tissues and cancer cell lines by real time RT-PCR.

J Endocrinol. 2001 Oct;171(1):R1-4.

PMID: 11572805 [PubMed - indexed for MEDLINE]

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Presentation Type: Consider for Oral Presentation

Basic Science Category: 8. Growth Hormone-Prolactin-Receptors & Actions

Awards: Travel Grant Awards.

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Title: REGULATION OF BCL-2 EXPRESSION BY HPRL AND ITS ANTAGONIST, HPRL-G129R, IN HUMAN BREAST CANCER CELL LINES

Susan K Peirce ^{1*}, Mike T Beck ¹ and Wen Y Chen ^{1,2}. ¹Dept. of Microbiology and Molecular Medicine, Clemson University, Clemson, SC, 29681 and ²Oncology Research Institute, Greenville Hospital System, Greenville, SC, 29605.

In previous studies, we have shown that the human prolactin antagonist hPRL-G129R is able to induce apoptosis in four human breast cancer cell lines. In the current study, a combination of PCR subtraction and cDNA microarray methodologies was used to examine the gene expression profiles of two estrogen receptor (ER) positive, and two ER negative human breast cancer cell lines following treatment with hPRL or hPRL-G129R. Among the many genes that were found to be differentially expressed, bcl-2 was strongly induced following hPRL treatment in three of the four cell lines tested. To confirm the evidence linking hPRL and bcl-2 expression, a quantitative method of real time RT-PCR and a Bcl-2 ELISA were used to measure bcl-2 mRNA expression levels and Bcl-2 protein levels, respectively. Eleven human breast cancer cell lines were assayed following treatment with hPRL, hPRL-G129R, or a combination of these proteins. We found that hPRL induced, while hPRL-G129R inhibited, bcl-2 mRNA expression in a majority of the cell lines tested, and that the induction of bcl-2 by hPRL was competitively inhibited by hPRL-G129R in most of these cell lines. The pattern of mRNA expression following these treatments correlated to the Bcl-2 protein levels. In particular, MCF-7, T47D and BT549 cells all demonstrated a three to four fold difference in bcl-2 expression levels between hPRL and hPRL-G129R treatments. In contrast, BT474, MDA-MB-231 and BT483 cells showed minimal bcl-2 response to hPRL or hPRL-G129R treatments. There was no correlation between estrogen receptor (ER) status and bcl-2 response to hPRL or hPRL-G129R treatment. The data from these studies suggest that hPRL increases the expression of bcl-2 message and Bcl-2 protein in some human breast cancer cell lines, and hPRL-G129R competitively inhibits bcl-2 expression induced by hPRL in these cell lines.

Supported in part by the Endowment Fund of the Greenville Hospital System and Grants (DAMD17-99-1-9129, DAMD17-01-1-0207, NIH/NCI 1R21CA87093).

Keyword 1: Prolactin antagonist

Keyword 2: Oncogene

Keyword 3: Breast cancer

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Basic Science Category: 8. Growth Hormone-Prolactin-Receptors & Actions

Awards: No award information selected.

Title: FROM AN ANTAGONIST BACK TO AN AGONIST: TWO WRONGS DO MAKE A RIGHT

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It is generally accepted that the initial step of signal transduction for human growth hormone (hGH) as well as human prolactin (hPRL) is to bind to their respective receptors. The binding process is reported to be sequential: one ligand binds to the first receptor through its binding site one with high affinity and then finds its second receptor through its binding site two with lower affinity resulting in a one ligand/two receptor complex. This ligand induced dimerization of the receptors is essential for hGH and hPRL signal transduction. Amino acid substitution mutation in binding site two of either hGH (hGH-G120R) or hPRL (hPRL-G129R) results in mutants with antagonistic effects both in vitro and in vivo demonstrated by many labs including ours. In our recent attempts to generate a more potent hPRL antagonist with a longer serum half-life, we produced a G129R-G129R homo-dimer using an E. coli expression vector, pET22b. The protein was purified using Q-sepharose anion exchange chromatography and a FPLC system. To our astonishment, the G129R-G129R homo-dimer acts in every aspect as an agonist assayed by STAT5 phosphorylation in human breast cancer cells. We found that the G129R-G129R homo-dimer is able to induce STAT5 phosphorylation in a concentration-dependent manner at a dose range similar to that of wild type hPRL. The induction of STAT5 phosphorylation is not only dose-dependent but also show self-antagonism in high concentration as seen in the case of hPRL. It is interesting to point out that the activation of STAT5 phosphorylation by G129R-G129R homo-dimer can be inhibited by G129R monomer. Our results suggest that as long as there are two binding sites (site 1 plus site 2 in wild type hPRL or site 1 plus another site 1 in G129R-G129R homo-dimer) in one molecule, the ligand serves as an agonist. Our data also suggests that the overall size of the ligand is not a crucial factor (23kd monomer or 46kd dimer) to induce PRL signal transduction. The potential use of homo-dimers of antagonists as longer half-life agonists needs further testing.

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